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Hibernation-Based Therapy to Improve Survival of Severe Blood Loss

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14. ABSTRACT
The purpose of these experiments was to address the dose response relationship for the toxicity of the drug product via I.V. or I.O. infusion and to assess the effectiveness of the drug to maintain adequate dosing concentrations when given either I.V. or I.O. We found no toxic effects from the drug when administered I.V. or I.O. as reported in pathology. Adequate dosing concentrations were maintained in animals receiving a high-dose of BHB/M. However, I.O. administration seemed to be more effective than the I.V. administration. When BHB/M was administered at full dose, I.V. administration was statistically more effective at maintaining the desired BHB/M concentrations when compared to the full dose BHB/M I.O. administration.

15. SUBJECT TERMS
Beta-hydroxybutyrate, melatonin, intra-osseous

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Table of Contents

Introduction:

Blast injuries have been responsible for the majority of combat deaths in Iraq and Afghanistan, and the likelihood of being exposed to explosives is increasing for military personnel and civilians alike in war zones and other regions of political conflict. The injuries sustained are often accompanied by severe blood loss, and shock from this blood loss is the most common cause of potentially salvageable deaths from combat related injuries.

D-beta hydroxybutyrate and melatonin (BHB/M) is a novel therapy designed to prolong survival in patients who are risk for bleeding to death. Our overall strategy in this series of studies is to use physiologic adaptive responses in hibernating mammals to aid in slavage of a patient with a potentially life-threatening blood loss, permitting survival to reach effective medical care. BHB/ M includes both an alternate fuel source for cells (D-beta hydroxybutyrate) and a powerful anti-oxidant, melatonin, to protect cells against damage.

Our goal is to evaluate BHB/M in animal models of injury that simulate the battlefield casualty. Our previous work has shown increased survival for both rats and pigs treated with BHB/M. We wish to prove that BHB/M is a safe and effective therapy that can decrease mortality and improve outcomes for injured casualties suffering from polytrauma and blast injuries.

Body:

Task 3: Assessment of BHB/M administered either intraosseously (IO) or intravenously (IV). 3 study groups: 1 dose based on Task 1, 6 animals/group (3 males and 3 females) and, n=36. Two sacrifice time points (day 2 and day 14), n=18 animals sacrificed on day 2 and n=18 animals sacrificed on day 14.

Task 3 utilized our pig model of tissue injury and hemorrhagic shock utilizing animals randomized based on the following experimental grid that was completed in January 2012 (Table 1). A timeline of the experimental protocol is located in the Appendix.

Table 1.

Treatment	Number of Animals Sacrificed	
	2 days	**14 days**
Lactated Ringers'	3 male, 3 female	3 male, 3 female
I.V. BHB/M @ 4M	3 male, 3 female	3 male, 3 female
I.O. BHB/M	3 male, 3 female	3 male, 3 female

We identified no statistical differences in either BHB or melatonin concentrations when comparing animals receiving the drug I.V. or I.O. (Figures 1 and 2), although both of these levels appeared to trend towards a lower level in animals treated with intraosseous BHB/M.

Figure 1. d-Betahydroxybutryate serum concentrations in animals undergoing injury and hemorrhagic shock. A trend towards higher levels in animals treated with I.V. BHB/M was noted, although this was not statistically significant.

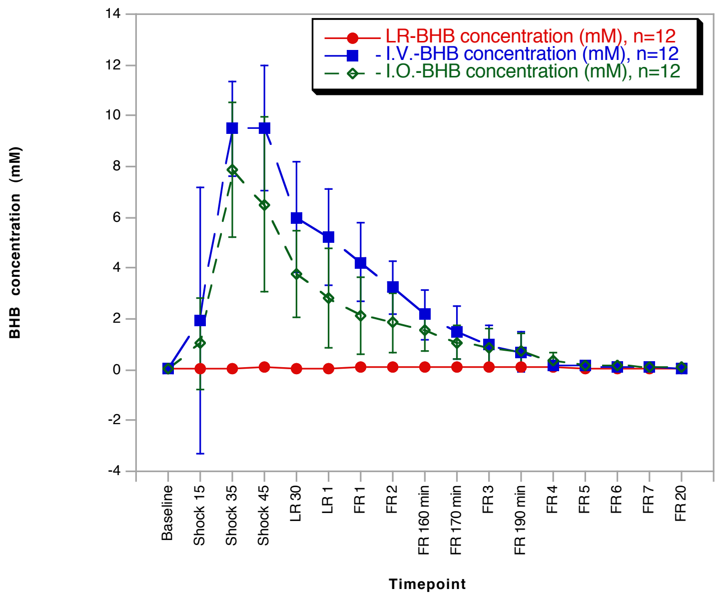
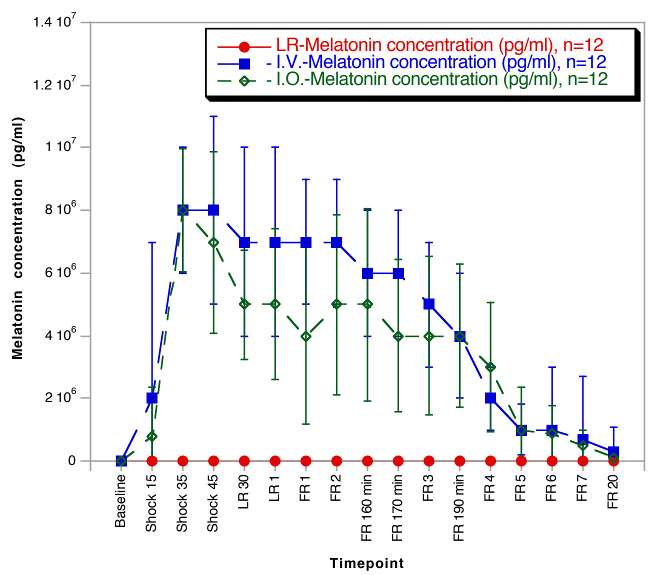
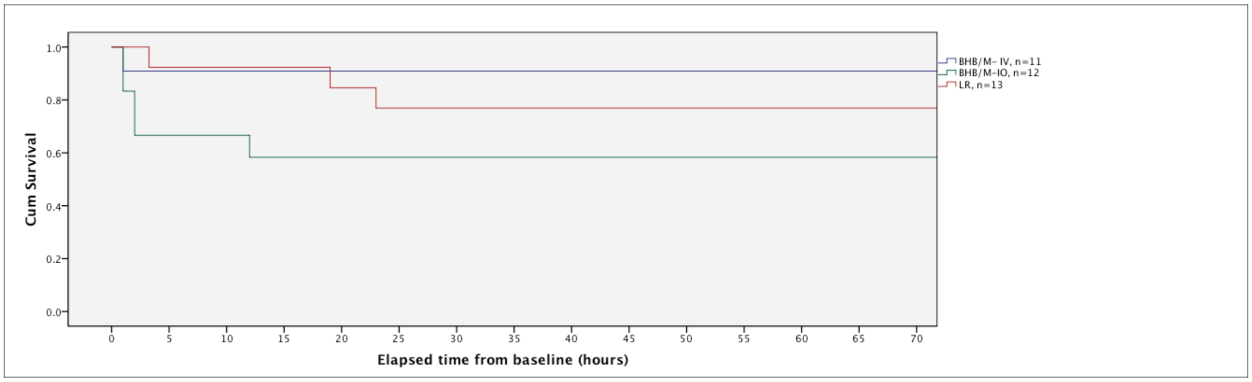


Figure 2. Melatonin serum concentrations in animals undergoing injury and hemorrhagic shock. A trend towards higher levels in animals treated with BHB/M was noted, although this did not reach significance.



Interestingly, survival to sacrifice did not differ significantly between groups. This appears to be related to lack of survival benefit in the group of animals who received I.O. BHB/M.

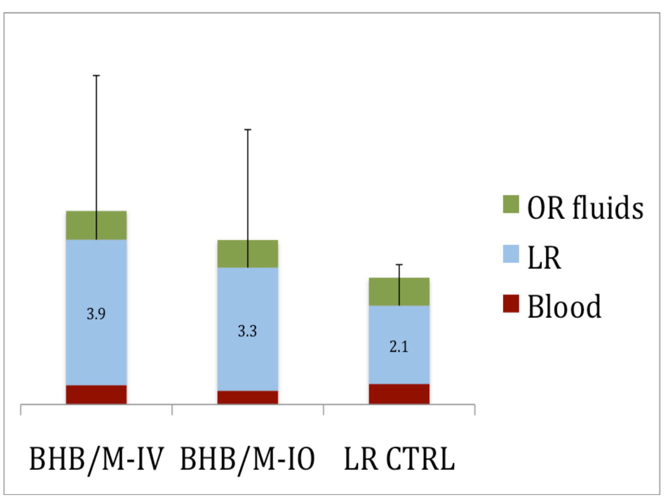
Figure 3. Kaplan-Meier survival curve of animals after injury and shock with standard resuscitation with or with our BHB/M infusion. p=0.171 when all groups compared, p= 0.132 I.V. compared to LR alone, p=0.673 I.O. compare to LR (chi-square test).



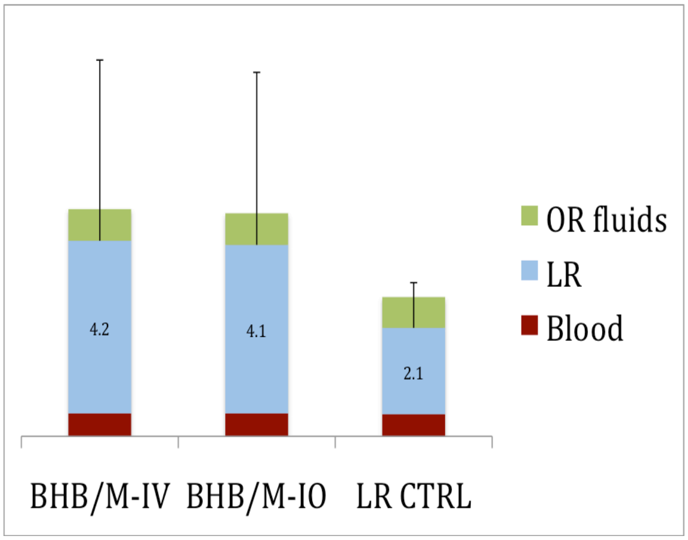
Fluid resuscitation reqirements did not differ between groups, either before or after censoring of animals who died with in 2 hours of initiation of resuscitation (Figure 4A and B).

Figure 4. Fluid administered during resuscitation in animals treated with BHB/M, either I.V. or intraosseously. A) All animals included in analysis, p=0.448 between groups. B) Excluding animals that died in the first 4 hours after shock and injury, p=0.299 between groups.

A)



B)



At the end of the experiments, necropsies were performed. The following tissue/organs were grossly inspected; adrenal glands, bones, brain, esophagus, eyes gall bladder, heart and great vessels, intestine both large and small, both left and right kidneys, liver, lymph nodes, left and right lung, oral cavity and tongue, pancreas, reproductive tract, skin, sternum bone marrow, stomach, thymus, thyroid, trachea and bronchi, urinary bladder and vessels. Histology was read from the following; heart, rib, tibia (the sight of intraosseous infusion of BHB/M), right and left kidney, right and left lateral liver lobe, thyroid gland, small intestine, large intestine, stomach gall bladder, adrenal glands, thymus pancreas, mesenteric lymph node, submandibular lymph node, urinary bladder, right and left lung, carotid artery, pituitary gland, cervical spinal cord, thoracic spinal cord, lumbar spinal cord, and brain. All necropsies and histology were performed/read either by George Ruth DVM, PhD, DACVP or Nick Robinson, BVSc, PhD, MACVSc, DACVP, Veterinary Pathologists contracted by Experimental Surgical Services.

There were no differences between groups in terms of histopathology. There were no detrimental effects noted at sites of intraosseous infusion of BHB/M.

We conclude from this work that there are no detrimental, histopathologic effects of BHB/M administered, either intra-venous or intra-osseous. This statement is true at sacrifice time-points, 72 hours and 14 days post-operatively (Table 2).

Table 2. Measured effects of BHB/Melatonin on safety/experimental measures in normal animals and animals after shock/injury protocol.

<table>
<tr><th>Measured Parameters</th><th colspan="4">Outcome</th></tr>
<tr><td>Coagulation Parameters
(Thrombolelastography, TEG)-Task 3 only</td><td colspan="4">Pending analysis</td></tr>
<tr><td>Liver Function
(ALT, AST, Albumin, total bilirubin, total protein)</td><td colspan="4">No differences</td></tr>
<tr><td>Renal Function
(urea nitrogen, creatine kinase, lactate dehygrogenase)</td><td colspan="4">No differences</td></tr>
<tr><td>Hematology
(hemoglobin)</td><td colspan="4">No differences</td></tr>
<tr><td rowspan="2">Histopathology
(heart, rib, tibia (the sight of intraosseous infusion of BHB/M), right and left kidney, right and left lateral liver lobe, thyroid gland, small intestine, large intestine, stomach gall bladder, adrenal glands, thymus pancreas, mesenteric lymph node, submandibular lymph node, urinary bladder, right and left lung, carotid artery, pituitary gland, cervical spinal cord, thoracic spinal cord, lumbar spinal cord, and brain)</td><td>Normal animals</td><td>~3.5 hours after infusion</td><td>72 hours after infusion</td><td>14 days after infusion</td></tr>
<tr><td colspan="4">No abnormal results were observed that were not consistent with injury/treatment.</td></tr>
<tr><td>Electrolytes
(Sodium, potassium)</td><td colspan="4">Reversible changes in sodium (elevation) and potassium (decrease) resolved with completion of infusion.</td></tr>
<tr><td>Physiology
(Heart rate, blood pressure, PA pressure, wedge pressure, urine output, temperature)</td><td colspan="4">No differences</td></tr>
</table>

Task 4: Task 4 was performed to further evaluate pharmacokinetics of IV and intraosseous administration of BHB/M. This is relevant especially given the finding that the intraosseous route at standard doses did not result in improved survival.

Pharmacokinetic assessment of BHB/M administered either intraosseous (IO) or intravenous (IV) was carried out using at various dosing regimens. 7 study groups with 4 animals/group (2 males and 2 females), n=28. One sacrifice time point FR 7.

Task 4 utilized a simple instrumentation procedure and drug infusion with animals randomized based on the following experimental grid beginning in February 2012 and completed in September 2012 (Table 2).

Table 2.

Group	Drug Component	Concentration of Drug component	Number of animals requested
1	Lacated Ringers'	10cc/kg, 1 cc/kg bolus 0.66 cc/kg/hr	4
2	BHB/M ½ dose I.V.	4 M BHB/ 43 mM melatonin 0.5 cc/kg bolus, 0.33 cc/kg/hr	4
3	BHB/M Full dose I.V.	4 M BHB/ 43 mM melatonin 1 cc/kg bolus, 0.66 cc/kg/hr	4
4	BHB/M Double dose I.V.	4 M BHB/ 43 mM melatonin 2 cc/kg bolus, 1.32 cc/kg/hr	4
5	BHB/M ½ dose I.O.	4 M BHB/ 43 mM melatonin 0.5 cc/kg bolus, 0.33 cc/kg/hr	4
6	BHB/M Full dose I.O.	4 M BHB/ 43 mM melatonin 1 cc/kg bolus, 0.66 cc/kg/hr	4
7	BHB/M Double dose I.O.	4 M BHB/ 43 mM melatonin 2 cc/kg bolus, 1.32 cc/kg/hr	4

I.V.= intravenous, I.O.=intraosseous

Melatonin and β-hydroxybutyrate (BHB) concentrations were obtained from the above outlined pharmacology experiments. Although the difference is not statistically significant, our results suggest that both melatonin and BHB concentrations are lower in animals receiving an intraosseous, full-dose infusion of BHB/M (green diamonds) when compared to the animals receiving an intravenous, full-dose infusion of BHB/M (blue triangles) (Figure 5 and 6). Interestingly, serum levels of both melatonin and beta-hyroxybutyrate in animals receiving BHB/M intraosseously, at full doses were similar to levels of animals receiving intravenous, half-dose.

We conclude that intraosseous infusion of BHB/M is associated with lower serum levels of both components at the currently utilized dose. The reason for this is unclear, but may be related to either bone marrow clearance or absorption, especially of the BHB component of the treatment. These decreased levels are likely below levels that we believe are associated with therapeutic effect (6-8mM).

Figure 5. Melatonin concentrations in animals receiving intraosseous and I.V. infusion of BHB/Melatonin at three doses compared to animals receiving lactated Ringers'. Animals were instrumented but did not undergo injury or shock protocol.

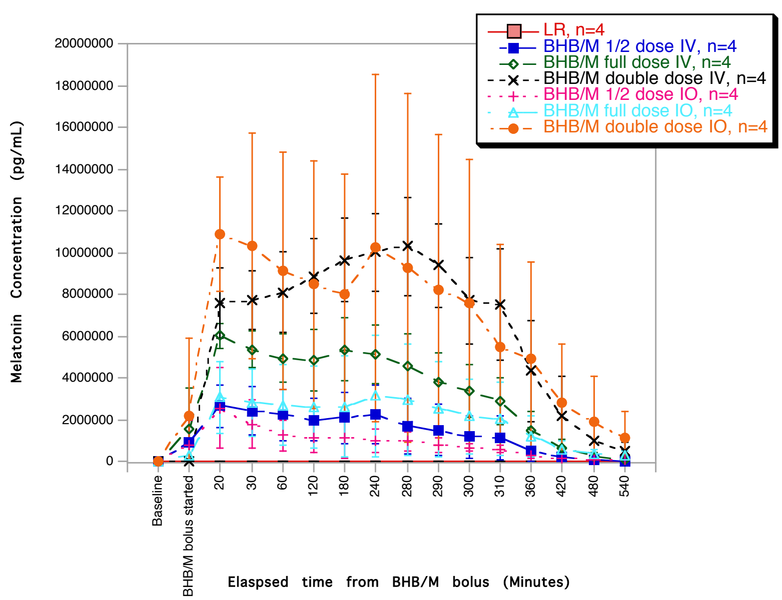
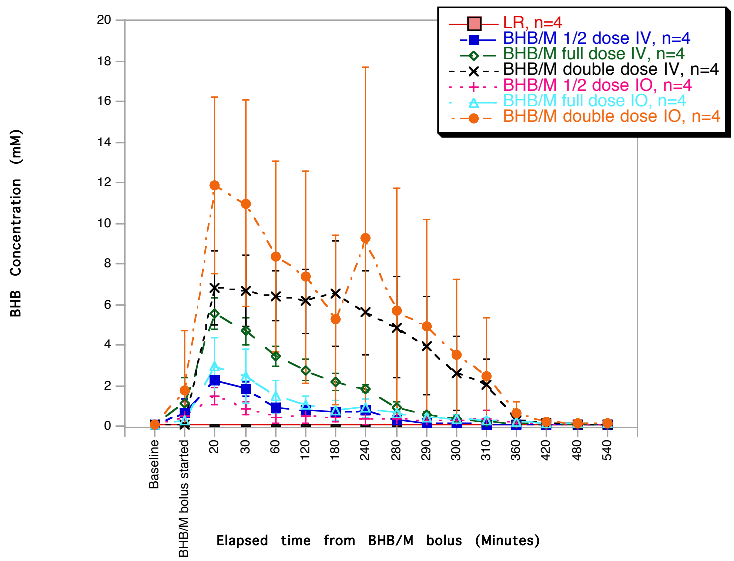


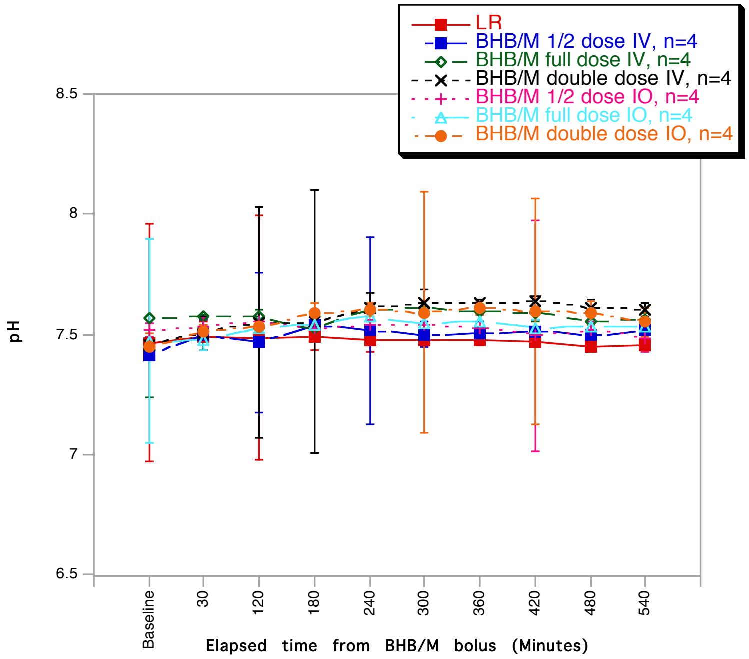
Figure 6. BHB concentrations in animals receiving intraosseous and I.V. infusion of BHB/Melatonin at three doses compared to animals receiving lactated Ringers'. Animals were instrumented but did not undergo injury or shock protocol.



Throughout the pharmacology arm of this study, we did not find any significant physiologic changes that occurred in animals receiving half dose, full dose, or double dose BHB/M. There were no deaths and no adverse events.

There were identifiable increases in pH in animals receiving BHB/M, with the highest pH reached of 7.635. This trend reached significance at two hours after infusion was started, and stayed significant throughout the infusion period for high doses. This effect resolved quickly after completion of infusion (Figure 7). We have previously described this effect (1). The cause is unclear. In a human experiment of infusion of BHB compared to glucose, BHB infusion was associated with an increase in bicarbonate production and a decrease in respiratory exchange ration (2). It is unclear whether this is an effect of the BHB on cellular metabolism, a chemical effect of the treatment, or an effect of this organic acid and renal tubular cells.

Figure 7.

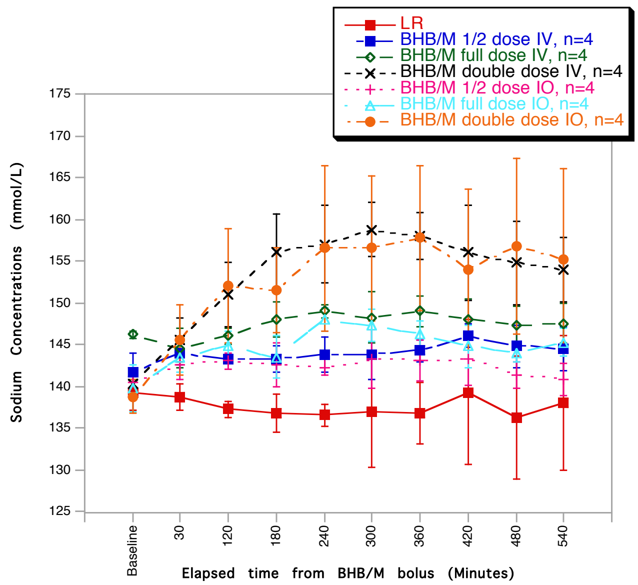


There were notable trends and differences in sodium concentrations in serum during infusion of double-dose BHB. At baseline, there were no significant differences in sodium levels between groups. Over the course of the experiment, there was a trend toward hypernatremia in the treatment groups, with the sodium levels in double-dose groups remaining above 150 mM/L one hour after bolus and infusion. The significant increase in sodium continued in the double-dose treated animals throughout the experiment, with double-dose groups having significantly higher sodium levels than the control group, as well as the half-dose I.O. group (Figure 8A).

There were also notable trends and differences in serum potassium levels during infusion. At baseline, there was no significant difference between groups. There is a noticeable trend during the initial post-infusion time points, for hypokalemia in the treatment groups. Beginning at one hour after bolus and infusion, there were significantly lower potassium levels in most experimental groups when compared to lactated Ringers'-infused, controls. This trend continues throughout infusion, with serum potassium returning towards baseline with interruption of infusion in all groups (Figure 8B).

Figure 8. Serum sodium and potassium levels in animals receiving varied dose of BHB/M compared to animals receiving infusion of lactated Ringers'. A) Serum sodium levels and B) serum potassium levels.

A)



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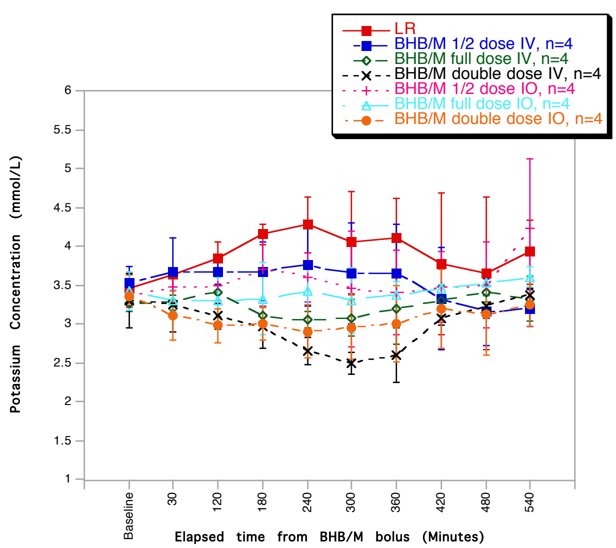

We conclude that the sodium and potassium changes are dose-related and trasient after administratuion of BHB/M. These changes were not associated with abnormalities in renal or cardiac function.

Of note, there were no significant differences between the groups found in the heart rate, cardiac output, oxygen requirements or delivery, blood pressure, or urine output. There was also no significant difference between groups in liver function tests, no change in BUN and no change in creatinine kinase. There was no laboratory evidence of damage or toxic effect to the function of major organs. Although there were notable swings in blood glucose levels, with intermittent difference being significant, there was no correlation of hypo- or hyperglycemia with changes in the drug dosage.

At the end of the experiments (~4.5 hours after the completion of infusion), necropsies were performed. The following tissue/organs were grossly inspected; adrenal glands, bones, brain, esophagus, eyes gall bladder, heart and great vessels, intestine both large and small, both left and right kidneys, liver, lymph nodes, left and right lung, oral cavity and tongue, pancreas, reproductive tract, skin, sternum bone marrow, stomach, thymus, thyroid, trachea and bronchi, urinary bladder and vessels. Histology was read from the following; heart, rib tibia, right and left kidney, right and left lateral liver lobe, thyroid gland, small intestine, large intestine, stomach gall bladder, adrenal glands, thymus pancreas, mesenteric lymph node, submandibular lymph node, urinary bladder, right and left lung, carotid artery, pituitary gland, cervical spinal cord, thoracic spinal cord, lumbar spinal cord, and brain. All necropsies and histology were performed/read either by George Ruth DVM, PhD, DACVP or Nick Robinson, BVSc, PhD, MACVSc, DACVP, Veterinary Pathologists contracted by Experimental Surgical Services. Pathology review in these animals confirmed the findings at our previous studies, with no significant abnormalities noted in BHB/M infused animals when compared with controls (Table 2).

Pathology for this task indicates that there are no detrimental effects when BHB/M is administered either intra-venous or intra-osseous in various doses.

Key Research Accomplishments:

* Completed our assessment of BHB/M administered either intraosseously (IO) or intravenously (IV).
* Completed assessment of BHB/M administered either intraosseous (IO) or intravenous (IV) at various dosing regimens.
* Are designing experiments that will reduce the concentration of melatonin thus reducing the amount of DMSO used to reconstitute melatonin. This series of experiment will also include group(s) of animals that will have higher dosing (4x and higher) using the current BHB/M formulation.

Reportable Outcomes:

* A manuscript entitled “BHB-M is Safe to Administer Peripherally “ is being prepared and will be sent to Journal of Surgical Research for peer review within the next two weeks.

Conclusion:

BHB/M at full dose given intraosseously did not result in improved survival compared with controls (Figure 3). This lack of effect appears to be related to blood levels of BHB (and potentially melatonin) given I.O. rather than I.V. (Figures 5 and 6).

BHB/M appears to be safe, when given both I.V. and I.O., with no identified histopathologic changes or physiologic changes associated with dose tested (Table 2).

References:

1. Mulier KE, Lexcen DR, Luzcek E, Greenberg JJ, Beilman GJ (2012) Treatment with beta-hydroxybutyrate and melatonin is associated with improved survival in a porcine model of hemorrhagic shock. Resuscitation 83(2):253-8

2. Chiolero R, Mavrocardatos P, Burnier P, Cayeux MC, Schindler C, Jeguier E, Tappy L (1993) Effects of infused sodium acetate, sodium lactate, and sodium beta-hydroxybutyrate on energy expenditure and substrate oxidation rates in lean humans. Am J Clin Nutr. 58(5):608-13.

Appendices:

Timepoints defined, Limited Resuscitation (LR)=maintenance of SBP above 80 mmHg, Full Resuscitation (FR)=maintenance of SBP above 90 mmHg, Hgb above 6 and Urine output > 1 cc/kg/hr.

Timepoint	**Elapsed time from Baseline**
Baseline	0
Shock 15	15 minutes
Shock 35	35 minutes
Shock 45	45 minutes
LR 30	30 minutes from the start of Limited Resuscitation phase, ~1.5 hours from baseline
LR 1	60 minutes from the start of Limited Resuscitation phase, ~2 hours from baseline
FR 1	1 hour from the start of Full Resuscitation, 2 hours from the start of Limited Resuscitation, ~3 hours from Baseline
FR 2	2 hour from the start of Full Resuscitation, 3 hours from the start of Limited Resuscitation, ~4 hours from Baseline
FR 160	160 minutes from the start of Full Resuscitation, 3 hours 40 minutes from the start of Limited Resuscitation, ~4.7 hours from Baseline
FR 170	170 minutes from the start of Full Resuscitation, 3 hours 50 minutes from the start of Limited Resuscitation, ~4.83 hours from Baseline
FR 3	3 hour from the start of Full Resuscitation, 4 hours from the start of Limited Resuscitation, ~5 hours from Baseline
FR 190	190 minutes from the start of Full Resuscitation, 4 hours 10 minutes from the start of Limited Resuscitation, ~5.2 hours from Baseline
FR 4	4 hour from the start of Full Resuscitation, 5 hours from the start of Limited Resuscitation, ~6 hours from Baseline
FR 5	5 hour from the start of Full Resuscitation, 6 hours from the start of Limited Resuscitation, ~7 hours from Baseline
FR 6	6 hour from the start of Full Resuscitation, 7 hours from the start of Limited Resuscitation, ~8 hours from Baseline
FR 7	7 hour from the start of Full Resuscitation, 8 hours from the start of Limited Resuscitation, ~9 hours from Baseline
FR 20	20 hour from the start of Full Resuscitation, 21 hours from the start of Limited Resuscitation, ~22 hours from Baseline